Early activated pathways responsible for CaP-induced bone formation in combination with human periosteum-derived cells

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Introduction: A strategy for bone tissue engineering is to combine osteochondroprogenitor cells, osteoconductive carriers and osteoinductive biomolecules in constructs that induce *in vivo* bone formation. During the past years, the field has been directed to implement *in vitro* processes that mimic *in vivo* tissue development. Therefore, when engineering these constructs, human periosteum-derived cells (hPDC) are a promising cell source. CaP is an interesting carrier source, as 70% of our bones consist of hydroxyapatite and certain CaP carriers are reported to be osteoinductive in combination with hPDCs. The identification of this bone forming mechanisms is a crucial step towards engineering robust constructs. It has been reported that bone formation in CaP-hPDC-containing constructs is dependent on the presence of Ca^{2+} together with the activation of Wnt and BMP-signalling, as deletion or inhibition of these parameters abrogated the bone formation. However, not much is known regarding the cellular mechanism of action in Ca-induced bone formation. Therefore, this study aimed to identify the early-activated cellular pathways that drive *in vivo* bone formation. In addition, we intended to identify the earliest time point and its associated important cellular and material variables which could be used to predict the bone forming capacity of CaP- and hPDC-containing constructs *in vivo*.

Materials & Methods: In order to investigate this, three commercially available CaP-carriers (CopiOs, VitOss and NuOss) known to have different bone forming capacities (0, 3 and 13% respectively) in combination with hPDCs, were seeded and implanted in an ectopic nude mouse model. To investigate activated signalling pathways, RNA and protein were isolated after both 24h of *in vitro* culture and 3 & 12 days of *in vivo* implantation, for gene expression analysis by quantitative PCR and protein phosphorylation by Western Blot. In parallel, Ca^{2+}-release from the carriers was measured during 21 days of *in vitro* incubation in PBS.

Results: After 24h, Ca^{2+}-release in CopiOs was 4-fold higher as compared to NuOss and 2-fold higher as compared to VitOss. In addition, after 3 days *in vivo*, a 20-fold higher phosphorylation could be seen. Gene expression analysis displayed upregulated Wnt-signalling in CopiOs-constructs after 24h *in vitro* (Axin2) and 3 days *in vivo* (LRP5). Gene expression for chondrogenic markers displayed a 5-fold upregulation of Sox9, a 2-fold higher ColII and a 6-fold higher Coll X expression in the NuOss carrier as compared to CopiOs after 12 days *in vivo*. Gene expression for bone markers displayed a 3-fold upregulation of Runx2 and over 20-fold higher expression of Osterix in the NuOss carrier as compared to CopiOs after 12 days *in vivo*. On protein level, BMP-signalling could be detected in the NuOss constructs by phosphorylation of Smad1/5/8 after 3 days *in vivo*, as compared to CopiOs constructs. This could be correlated to upregulated expression of BMP-ligands after 24h *in vitro* (BMP-7) and 3 days *in vivo* (BMP-2 and BMP-7). In addition, a more than 2-fold higher expression of BMP-target genes ID1 and ID3 was found in the same constructs. By hierarchical clustering, factors could be grouped based on their dissimilarity. Those factors who had a strong effect on the *in vivo* bone forming capacity were further evaluated by partial least square modelling, to predict the *in vivo* bone formation based on the day 12 samples. Variables with high importance were BMP-2, Runx2, Sox9 and ID1 expression.

Conclusion: In conclusion, this study highlights the crucial inhibitory effect of a high Ca^{2+}-release (via phosphorylation of PKC and subsequently β-catenin) on CaP-induced bone formation, which abrogates early activated Wnt signalling. In the presence of a lower Ca-release, early BMP-activation initiated bone formation, which later was driven by enhanced BMP-signalling and followed by stimulated Wnt-signalling. Suitable prediction markers were found to be BMP-7, Runx2, Sox9 and ID1 expression in explants isolated 12 days after *in vivo* implantation.